International Journal of Pharmaceutics, 32 (1986) 55–60
Elsevier

IJP 01063

Difference of *Pseudomonas aeruginosa* sensitivity to chloroxylenol according to the culture medium

M.J. Devleeschouwer, P. Boussard, P. Momin and J. Dony

Laboratoire de Microbiologie et d'Hygiène CP 205/2, Institut de Pharmacie, 1050, Brussels (Belgium)

(Received December 10th, 1985)
(Modified version received March 23rd, 1986)
(Accepted March 28th, 1986)

Key words: *Pseudomonas aeruginosa* – chloroxylenol – permeability – outer-membrane

Summary

The authors recorded notable difference of sensitivity of *Pseudomonas aeruginosa* to chloroxylenol according to the growth medium; the amount of magnesium of the culture medium and the growth phase were not major factors. This difference, which can be extended to various strains, is due to a difference of permeability of the outer membrane. It is suggested that adsorption of medium components on the surface of the bacteria could participate in the phenomenon. Similar results were obtained with phenol and crystal violet.

Introduction

As shown in a previous publication (Dony et al., 1984), we recorded notable sensitivity variations of *Pseudomonas aeruginosa* ATCC 15442 to some disinfectants according to the medium in which the organism was cultured before the contact with the antimicrobial agents. In particular, *Pseudomonas aeruginosa* is much more sensitive to the action of chloroxylenol when the strain is grown in brain heart infusion (B.H.I. (Difco)) rather than in tryptic soy broth (T.S.B. (Difco)).

It is known that the Gram-negative bacteria and especially *Pseudomonas aeruginosa* are outstandingly resistant to antibacterials on account of the impermeability of their outer membrane (Leive, 1974; Yoshimura and Nikaïdo, 1982; Nikaïdo and Vaara, 1985). Thus for a disinfectant to be active it must first cross the outer membrane to reach the cytoplasmic membrane and act on the microbial cell. The difference of sensitivity to chloroxylenol according to the culture medium used to prepare the inoculum could thus be linked to a greater 'permeability' of the organism grown in brain heart infusion (B.H.I.) relative to the one cultured in tryptic soy broth (T.S.B.). The fact that this difference is abolished by the addition to chloroxylenol of EDTA, which disorganizes the outer-membrane, supports this hypothesis (Dony et al., 1984).

Our former investigations were carried out on the single strain *Pseudomonas aeruginosa* ATCC 15442 which has been recommended for the in vitro evaluation of disinfectants (Devleeschouwer and Dony, 1981); it seemed important therefore

Correspondence: M.J. Devleeschouwer, Laboratoire de Microbiologie et d'Hygiène CP 205/2, Institut de Pharmacie, Université de Bruxelles, Campus de la Plaine, Boulevard du Triomphe, 1050 Brussels, Belgium

first to evaluate how the observed divergence according to the growth conditions appeared for other strains of *Pseudomonas aeruginosa*. We therefore reproduced the experiments with other collection strains and with strains isolated in hospital. The general character of the observation being demonstrated we then carried on our investigations with the single *Pseudomonas aeruginosa* ATCC 15442 strain.

In connection with the hypothesis of a modification occurring during growth it seemed important to investigate a factor well known to influence the activity of antibacterials on *Pseudomonas*, namely the magnesium content of the two culture media (Hancock et al., 1981; Takeuchi and Nikaïdo, 1981).

We also verified whether we could record with phenol and crystal violet the same alteration of the sensitivity of *Pseudomonas aeruginosa* as that obtained with chloroxylenol according to the media used for the preparation of the inoculum. Indeed, it is well known that phenol and crystal violet, lipophilic compounds, use the hydrophobic pathway to cross the outer-membrane of *Pseudomonas aeruginosa* (Kropinski et al., 1978; Hancock, 1984). Chloroxylenol, which is a little hydrophobic molecule, should act like phenol. The results obtained with phenol, cresyl violet and chloroxylenol will enable us to orientate more precisely our future work on the role of the outer membrane permeability in the sensitivity of *Pseudomonas aeruginosa* to several antimicrobials.

Materials and Methods

The method for evaluation of the in vitro bactericidal activity of disinfectants has been described in a previous publication (Devleeschouwer and Dony, 1981). This method was simplified, no addition of bacteria being performed after the first contact time between the disinfecting solution and bacteria. The bactericidal activity is determined by measuring the logarithmic reduction of the initial number of organisms after a contact time of ten minutes with the disinfectant solution. Furthermore, the contact time between the disinfectant solution and the bacteria is extended for a period of 10 days. A bacterial count is then performed to assess if after the initial decrease of the number of organisms the eventual survivors are able to multiply in the disinfecting solution within this period.

Culture media

Medium A: tryptic soy broth (Difco)

Tryptone	17 g
Soytone	3 g
Dextrose	2.5 g
Sodium chloride	5 g
Dipotassium phosphate	2.5 g
Distilled water	1000 ml

Autoclaved for 15 min at 121°C.

Medium B: brain heart infusion (Difco)

Calf brain (infusion from)	200 g
Beef heart (infusion from)	250 g
Proteose peptone	10 g
Dextrose	2 g
Sodium chloride	5 g
Disodium phosphate	2.5 g

Dissolve 37 g in 1000 ml distilled water and autoclave for 15 min at 121°C.

As described in our previous publication (Devleeschouwer and Dony, 1981), continuous cultures of the strain must be made by daily transfer on H.I.A. (heart infusion agar) or T.S.A. (tryptic soy agar); slants and trials can only be performed after three subcultures. We then studied the influence of the number of subcultures on the responsiveness of the organism to chloroxylenol. The 5th subculture gave the greatest difference of responsiveness between the two media. After the 8th subculture no further difference could be recorded. We then added to use the strain only between the 4th and 6th subculture to perform our experiments.

Investigated strains

The *Pseudomonas aeruginosa* ATCC 15442 was used as test strain during all the experiments. Two other collection strains, namely *Pseudomonas aeruginosa* ATCC 9027 and ATCC 6749, and four hospital strains isolated during infectious episodes were used to verify if the recorded difference

could be extended to other *Pseudomonas aeruginosa* strains.

The analysis of the mode of action of chloroxylenol on *Pseudomonas aeruginosa* makes obvious the importance of the bonds assumed by the bivalent cations in the external wall structures and the consequential interaction of EDTA (Eagon and Carson, 1965; Gray and Wilkinson, 1965a and b; Cox and Eagon, 1968; Brown and Melling, 1969). Based more specifically on the work of Dankert and Schut (1975) showing the influence of the magnesium content of the culture medium on the sensitivity of *Pseudomonas aeruginosa* to chloroxylenol and the potentiation by EDTA of the disinfecting action, we determined the amount of magnesium in the two culture media by atomic absorption using a flame spectrophotometer Varian Techtron AA-5. The reagent blanc was the diluting solution containing, 3.88g strontium chloride and 9 ml perchloric acid in 1 litre of twice-distillated water. The analyzed samples contained 0.1 ml of the culture medium to be assayed with 4.9 ml diluting solution to which 0, 0.1, 0.2 or 0.4 μg/ml magnesium were added.

We then comparatively followed the growth curve in the two media to verify that the bacteria were in the same growth phase before their introduction into the disinfecting solution. One knows indeed that the bacteria can have different sensitivities when they are in the exponential logarithmic phase or in the stationary phase. The growth curves of *Pseudomonas aeruginosa* ATCC 15442 were established by measuring at various times the optical density by means of a spectrophotometer (Spectronic 20, Bausch and Lomb) of the bacterial suspension in both media.

In connection with the hypothesis of a permeability alteration resulting from the adsorption of medium components on the bacterial external structures we carried out the following experiment: bacteria growth in T.S.B. were transferred after washing in B.H.I. and after a contact time excluding their multiplication (passive contact), they were exposed to the action of the disinfectant. Inversely, bacteria grown in B.H.I. were exposed to the action of the disinfectant after a passive contact in T.S.B. When starting, we used an inoculum grown in our normal conditions (4–6 preliminary subcultures on agar slants followed by 16 h at 37°C in liquid medium); 30 ml of the culture were centrifuged for 10 min at 3000 rpm. The cells were then washed 3 times with normal saline or used without washing. The organisms were suspended in 30 ml of the medium not used for the initial preparation of the inoculum for a contact time of 40 min at room temperature or 1 h at 37°C. Thereafter a normal capacity test was performed. Controls were made with uncentrifuged cells or in some cases resuspended in normal saline after centrifugation.

When studying the sensitivity of *Pseudomonas aeruginosa* to crystal violet, we used the maximal available concentration of 12 mg/ml. In the case of phenol concentrations of 0.25%, 0.75% and 1% were used in the capacity test.

Results

Influence of the nature of the strain on the difference of action of chloroxylenol on Pseudomonas aeruginosa

The results of the determination of the effectiveness of chloroxylenol 0.245% according to the strain and the culture medium are summarized in Table 1. It is evident that in most cases the difference of sensitivity between the two media exceeds 2 and more log phases in favour of the brain heart infusion. Even when the results in tryptic soy broth are more favourable and even reach a 5 log diminution and when the difference between the two media is about 1.5 log phase, it is not possible to count any survivors in brain heart infusion when extending the contact between bacteria and disinfectant solution for a longer time. In contrast, bacteria grown in tryptic soy broth did not completely die and survivors were able to regrow attaining levels as high as 10^6 organisms/ml after 10 days.

Magnesium content of the two media

It is possible to verify experimentally that the magnesium level of the two media used is not the reason for the difference of sensitivity of the bacteria by measuring the concentration of this cation in the two media by atomic absorption. The

TABLE 1

SENSITIVITY OF SEVERAL *PSEUDOMONAS AERUGINOSA* STRAINS TO CHLOROXYLENOL 0.245%

Strain	With serum					Without serum				
	TSB*	BHI*	BHI-TSB***	TSB** (10 days)	BHI** (10 days)	TSB*	BHI*	BHI-TSB***	TSB** (10 days)	BHI** (10 days)
ATCC 15442	3.84	> 6.12	> 2.28	–	–	4.51	> 6.16	> 1.65	–	–
	5.54	7.35	1.81	10^6	0	5.98	> 8.09	> 2.29	10^5	0
	4.63	> 7.03	> 2.40	10^6	0	5.49	> 7.07	> 1.58	0	0
	2.30	5.40	3.10	–	–	2.90	6.10	3.20	–	–
ATCC 6749	< 4.11	7.31	> 3.20	10^6	0	5.03	6.66	1.63	10^6	0
	4.5	> 6.77	> 2.27	10^6	0	5.53	7.12	1.59	10^6	0
	3.62	> 7.49	> 3.87	10^6	0	< 4.36	6.83	> 2.47	10^6	0
	< 3.11	5.03	> 2.19	–	–	3.62	5.20	1.58	–	–
ATCC 9027	< 3.30	5.48	> 2.18	–	–	3.81	5.34	1.53	–	–
	4.60	6.40	1.80	–	–	4.70	7.30	2.60	–	–
Hosp 1 (1010)	< 3.92	7.10	> 3.18	–	–	< 3.96	6.84	> 2.88	–	–
Hosp 2 (1008)	< 3.48	5	> 1.52	–	–	< 3.51	5	> 1.49	–	–
Hosp 3 (614)	4.02	6.81	2.79	10^6	0	4.92	7.85	2.83	10^6	0
Hosp 4 (722)	3.41	> 7.18	> 3.77	10^6	0	< 4.25	> 7.39	> 3.14	10^6	0

* Reduction of the number of bacteria/ml expressed as logarithms.

** Number of bacteria/ml after 10 days of contact between bacteria and disinfectant.

*** Difference in log reduction between the two media.

TABLE 2

RESULTS OF THE PASSIVE TRANSFER EXPERIMENTS, CHLOROXYLENOL 0.245% *PSEUDOMONAS AERUGINOSA* ATCC 15442

Medium	Serum +	Serum −	Serum +	Serum −	Serum +	Serum −	Serum +	Serum −
BHI	3.2*	4.2	2.6	2.8	> 7.76	> 7.79	> 6.12	> 6.16
TSB	1.5	2.6	1.0	1.5	4.39	5.12	3.84	4.51
TSB → BHI	3.0	3.1	2.8	2.6	> 7.46	> 7.50	> 6.36	5.49
BHI → TSB	2.7	2.9	2.5	2.9	> 7.23	6.26	5.49	6.46
Contact	40 min at 20°C		40 min at 20°C		1 h at 37°C		1 h at 37°C	
Washing	3 times						no washing	

* Reduction of the number of bacteria/ml expressed as logarithms.

TABLE 3

INFLUENCE OF THE CULTURE MEDIUM ON THE SENSITIVITY OF *PSEUDOMONAS AERUGINOSA* ATCC 15442 TO PHENOL AND CRYSTAL VIOLET

	with serum			without serum		
	TSB	BHI	≠ BHJ-TSB	TSB	BHI	≠ BHI-TSB
Cresyl violet (12 mg/ml)	−0.2*	0.8	1	−0.1	1.2	1.3
Phenol 0.25%	−0.4	0.8	1.2	−0.4	2.0	2.4
Phenol 0.75%	0.3	1.3	1	1	1.7	0.7
	0.2	1.1	1.3	0.1	1.4	1.3
Phenol 1%	2.3	4.4	2.1	3.1	6.0	2.9

* Reduction of the number of bacteria/ml expressed as logarithms.

assays showed that the medium A contained 14.05 μg of Mg^{2+}/ml (= 0.57 mM), and medium B 12.18 μg of Mg^{2+}/ml (= 0.501 mM). In our opinion this difference was not sufficient to explain the sensitivity modifications according to the medium.

Growth curves of Pseudomonas aeruginosa *ATCC 15442 in the two culture media*

The growth of *Pseudomonas aeruginosa* is initially more important in brain heart infusion, but after 18 h of culture, the time when the inoculum is used for the efficacity testing, the bacteria are in the same stationary phase and are thus able to respond in the same way to the antibacterial agent.

Results of the passive transfer experiments

The results of the passive transfer experiments are summarized in Table 2. The transfer after centrifugation in brain heart infusion of organisms, which were originally grown in tryptic soy broth, always resulted in the modification of the sensitivity to chloroxylenol. The bacteria were sensitive to chloroxylenol to the same extent as bacteria grown in brain heart infusion.

Influence of the culture medium on the sensitivity of Pseudomonas aeruginosa *ATCC 15442 to phenol and crystal violet*

The data from the experiments concerning phenol and crystal violet are summarized in Table 3. The organisms are more sensitive in brain heart infusion: the difference between the two media always being greater than 1 log unit.

Discussion and Conclusions

Our data suggest that the difference of sensitivity of *Pseudomonas aeruginosa* to chloroxylenol according to the growth medium can be extended to various strains. The amount of magnesium of the culture medium does not seem to be a major factor unlike the growth phase, the bacteria being in the stationary phase. The difference of permeability could be due to the fact of a modification of the outer-membrane of the bacteria during growth. But it is conceivable that the alteration of permeability simply arises from the adsorption of medium elements on external structures of the bacteria. The 'passive transfer' experiments suggest that adsorption in brain heart infusion medium components on the surface of the bacteria grown in tryptic soy broth could participate in the enhancement of sensitivity of *Pseudomonas aeruginosa* to chloroxylenol. However, the influence of other factors involved complementarily in the biosynthesis steps (e.g., peptones, phospholipids) cannot be ignored. Finally, the similar results recorded with the two media for chloroxylenol, phenol and crystal violet is of particular interest. It may be concluded by analogy that the permeability through the external wall is the factor conditioning the difference of sensitivity according to the culture medium. It should be remembered that for *Pseudomonas aeruginosa*, one can characterize three uptakes across the outer membrane (Caulcott et al., 1984 Hancock, 1984; Nikaido and Vaara, 1985).

Chloroxylenol should take the pathway specifically reserved for hydrophobic molecules such as phenol and cresyl violet and the crossing of the external wall of this molecules should be promoted by components appearing only in brain heart infusion and that could be fixed on the external wall.

It is indeed well known that the lipopolysaccharides (LPS) draw up a homogeneous frame owing to the cross-linkings between adjacent LPS molecules. This structure is stabilized by bivalent cations. The resistance to some hydrophobic molecules would seem to be due to particularly stable lipopolysaccharides; every destabilization of the LPS could promote the permeability of the external wall.

Perhaps it could be possible to consider the sensitivity promoting factors to find cases which could increase the in vivo permeability of the bacterial cell.

Acknowledgements

This work was made possible to C.S.T. Contract Nr 20674. We thank Martine Otten for her excellent technical assistance.

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